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EFFECTS OF ECDYSTERONE ON THE DEPOSITION OF COCKROACH CUTICLE *IN VITRO*

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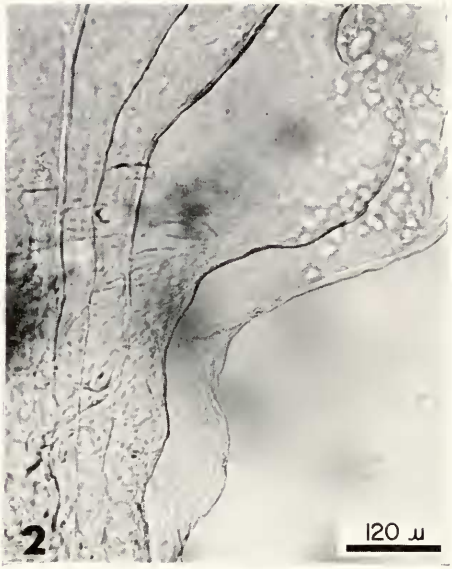
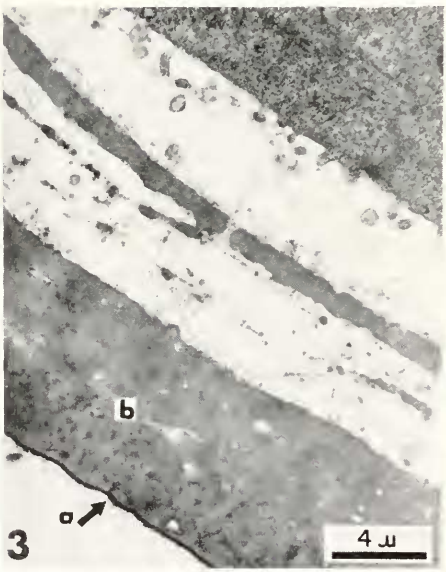
The production of cuticular materials by insect tissue *in vitro* in response to stimulation by the molting hormone was discussed in a recent review (Marks, 1970). In these early studies, relatively large amounts (3 to 25 μg/ml of medium) of the hormone were used, the period of exposure ranged from 2 to 10 days, and no systematic attempt was made to determine the effects of different doses. In a recent study, Marks and Leopold (1971) reported that cuticle deposition in cockroach leg regenerates could be induced *in vitro*. High doses (2 to 25 μg/ml) given over long periods (7 to 10 days) resulted in the deposition of a heavy cuticle complete with well-defined setae; smaller doses produced a thinner cuticle on which droplets of chitin-bearing material accumulated. The lowest concentration that induced any deposition was 0.1 μg/ml given over a period of 7 days. These results suggested that a quantitative study of hormonal induction of cuticular deposition *in vitro* might facilitate the investigation of the mechanisms whereby ecdysterone activates the epidermal cells and of the process of cuticle deposition.

In the present study, the effects of three dose-related variables were investigated: the concentration of the hormone, the duration of exposure, and interruption of the dose.

Methods

Late-instar nymphs of the Madeira cockroach, *Leucophaca maderae* (F.) were removed from the laboratory colony immediately after molting. The mesothoracic legs were removed 24 hr later at the femora-trochenteral joint. The operated insects were held for 24–26 days at room temperature in paper cups and fed dog food and water. At the end of this period, the coxae were dissected, and the regenerating legs, which at this stage comprised about 2 mm^3 of tissue each, were removed and placed in multipurpose tissue chambers (Rose, 1954) filled with 2 ml of M20 nutrient medium containing 5% fetal bovine serum. These were maintained at 27° C. The ecdysterone dissolved in water (1.0 or 0.1 μg/μl) was held under refrigeration, and a new solution was made up every 30 days. The hormone was injected into the chambers, and at the end of the period of exposure, the treated medium was removed and the chambers were rinsed twice and refilled with fresh medium. All chambers were observed periodically for 14 days after exposure to the hormone and then scored for the presence of cuticle. Since the thickness of the cuticle varied with the dose, a positive score was given only if both chitin droplets and a cuticular membrane were present on at least two areas of the explant (Marks and Leopold, 1971).





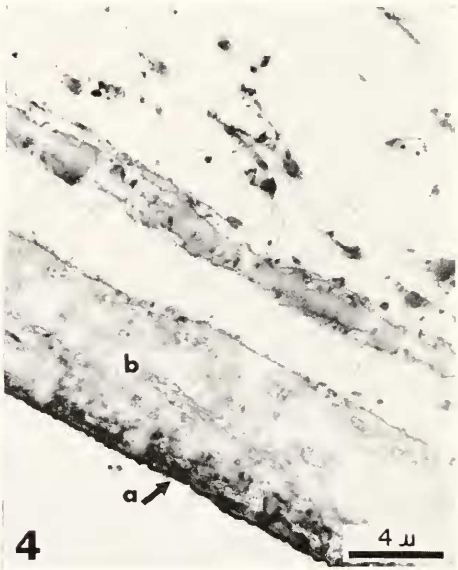


FIGURE 1. Leg regenerate with five successive cuticles resulting from five separate doses of ecdysterone. Secretion of most recent cuticle is still incomplete, and visualization is obscured by tissue.

FIGURE 2. Same specimen as Figure 1 after epidermis has retracted. Most recent cuticle is complete, and five cuticles are clearly visible.

FIGURE 3. Electron micrograph of inner of two cuticles secreted by a single leg regenerate. Surface of epidermal cell is visible at upper right. Epicuticle (a) is clearly distinguishable from less dense procuticle (b).

TABLE I

Comparison of responses obtained by administering a 1-μgd dose of ecdysterone in different ways

Number of exposures	Length of exposure (days)	Dose (μg/ml)	Total dosage (μgd)	Total days		Frequency of response (%)
				Exposed	Elapsed*	
1	1	1.00	1	1	1	16
2	1	0.50	1	2	5	25
4	1	0.25	1	4	13	20

* Includes time between repeated doses.

Two types of dosage were investigated, those in which the period of exposure was constant and the concentration of the hormone was varied and those in which the concentration was constant and the exposure was varied. Also, two types of exposure were used, continuous and interrupted. For the interrupted exposure, the hormone was injected for a given period and then the chambers were rinsed, refilled, and held for 3 days before the next exposure; this procedure was repeated until the total dose was given. The total dose received by an explant was expressed as the number of days of exposure multiplied by the concentration of the hormone in μg/ml of medium. Thus, the expression of dosage in microgram days (μgd) was common to all the experiments.

RESULTS

Since time-dose studies often involve long exposures (or as with interrupted doses, long periods during which the total dosage is delivered), it was necessary to establish that an effective dose of the hormone was maintained under the conditions of the experiment and that the explant retained its sensitivity to the hormone over the entire period of exposure. Therefore, three chambers, each containing two explants, were injected with ecdysterone to give a final concentration of 2.5 μg/ml and incubated for 30 days. At the end of this period, all the explants had well-developed cuticle. The medium was then removed from these chambers and injected into other chambers containing fresh explants. Cuticle appeared on all the new explants within 10 days. In another experiment, ecdysterone (2.5 μg/ml) was injected into a chamber containing two explants. The medium from this chamber was transferred after 7 days to another containing two more fresh explants and transferred to a third chamber after 7 more days. Then after 7 days in the third chamber, the medium was returned to the first chamber for 7 days (no new chambers were available) and finally on again to the second one. Within 10 days after the first dose, the explants in all three chambers showed evidence of cuticular deposition. Furthermore, when the explants were exposed to the medium the second time, they developed a second cuticle inside the first. Moreover, when a chamber containing fresh explants was injected with the aged

FIGURE 4. Electron micrograph of outer cuticle of same specimen as in Figure 3. Again, epicuticle (a) can be distinguished from procuticle (b). Similarity in the structure of the two cuticles is apparent.

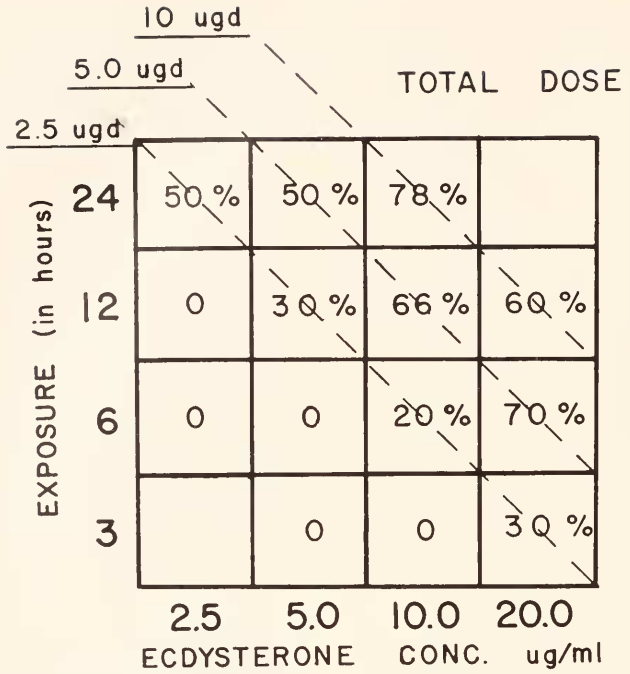


FIGURE 5. Diagram showing relative effect of concentration of hormone, time of exposure, and total dose (μgd) received on the frequency (%) of cuticle deposition by cockroach leg regenerates treated *in vitro* with ecdysterone.

medium some 35 days after the start of the experiment, cuticle appeared within 10 days. Superficially at least, this cuticle was no different from the first one in the series. The same experiment was repeated with a lower total dose (0.5 μg/ml for 5 days). After passage through three chambers, enough ecdysterone still remained in this medium to induce cuticular deposition on fresh explants in a fourth chamber.

Apparently neither metabolism by as many as 10 regenerates nor exposure of the hormone to breakdown over a period of 30 days was sufficient to reduce the dosages to a level below the threshhold. Marks and Leopold (1971) demonstrated that untreated regenerates used as solvent (H_2O) controls could be induced to respond to ecdysone treatment, even after 14 days *in vitro*. This ability to respond over a period of time was further explored by treating a series of chambers with ecdysterone (2.5 μg/ml). After five days, the treated medium was removed, and the chambers were rinsed and refed. As soon as cuticular deposits appeared on the explants, the chambers were refed and retreated with ecdysterone. This cycle was repeated until the tissues failed to respond. In this way, we were able to obtain as many as five separate cuticles from a single explant (Figs. 1 and 2), and the tissues apparently retained the ability to respond to treatment over 50 days.

One explant that had produced two cuticles was examined by electron microscopy: A section of the inner (second) cuticle (Fig. 3) was compared with a

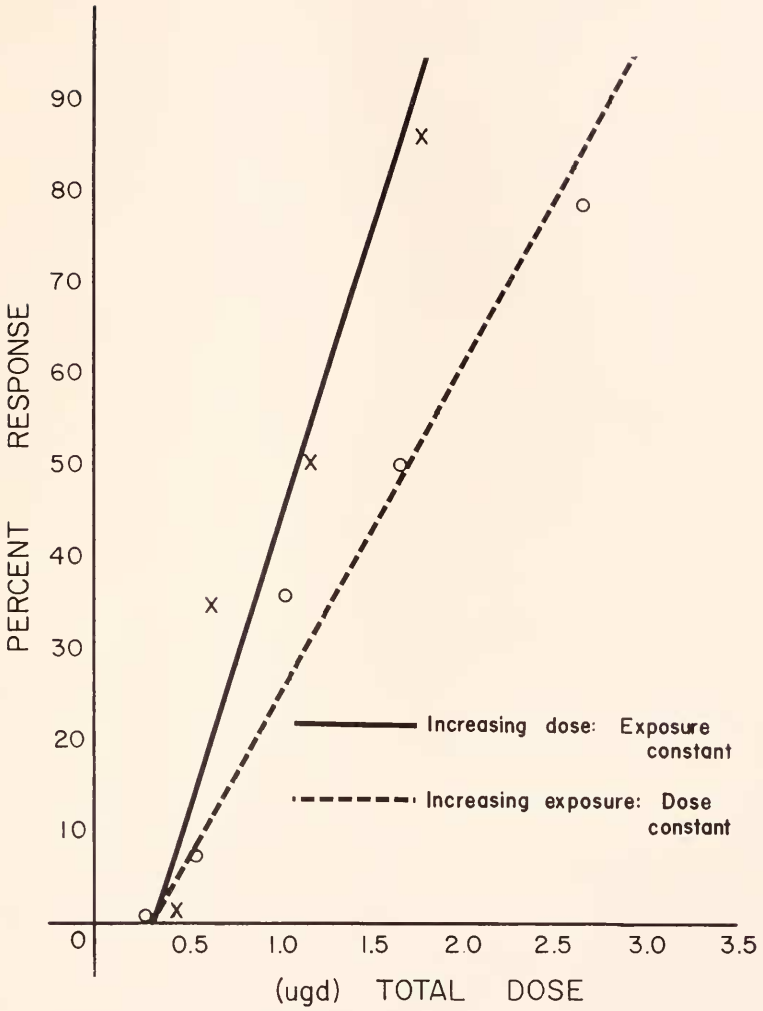


FIGURE 6. Graph showing the effect of the mode of delivery of a given total dose of ecdysterone on the frequency of cuticle deposition in cockroach leg regenerates.

section of the outer (first) cuticle (Fig. 4). Except for their position relative to the explant, it was difficult to distinguish between them. Each cuticle had an epicuticle (a) and a procuticle (b), evidence that they were the products of separate secretory cycles.

Once the long-term stability of the experimental system was established, a series of time-dosage tests was made. In the first series, high concentrations of ecdysterone (2.5 to 20 μg/ml) and short exposures (3 to 24 hr) were used. The minimum number of replicates for each determination was 10, and the results are given in Figure 5. The response to the hormone could be increased by increasing either the concentration or the length of exposure and was reasonably consistent in

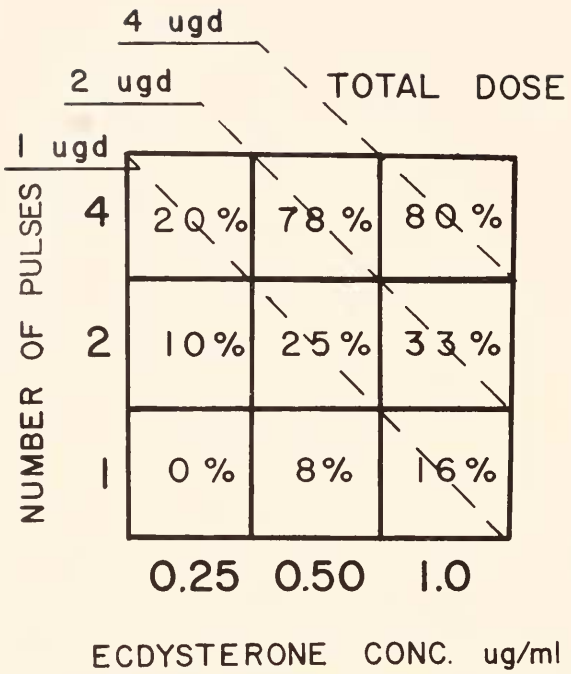


FIGURE 7. Diagram showing the relative effect of hormone concentration, number of exposures, and total dose received on the frequency (%) of cuticle deposition by cockroach leg regenerates treated *in vitro* with ecdysterone.

terms of total dose (μgd). When the exposure was 12 hr or less, the 50% response level lay between 2.5 and 5 μgd. At shorter intervals, the ecdysterone concentration required became so high, further experiments were impractical.

In the second series, the concentration of ecdysterone was lowered, and the period of exposure was increased. In one experiment, the period of exposure was held constant at 7 days, and the concentrations of ecdysterone varied from 0.05 to 0.50 μg/ml. In another experiment, the concentration was held constant at 0.50 μg/ml, and the periods of exposure varied from 12 hr to 7 days. The minimum number of replicates used to obtain each point was 10, and the results are given in Figure 6. The data show that time of exposure and concentration of hormone make roughly equal contributions to the effectiveness of the total dose. The dose required to give a 50% response lay between 1.0 μgd with a 7-day exposure and 1.4 μgd with a 3-day exposure. This was roughly half the dosage required for the short-term experiments.

Since the preliminary experiments showed that repeated large doses (10 μgd or greater) produced a series of separate cuticles, an additional series of experiments was made with repeated exposures of 1 day and concentrations of 0.25, 0.50, and 1.0 μg/ml. The minimum number of replicates was 12. After each dose, the chambers were washed with 3 changes of nutrient and held 3 days before the dose was repeated; as many as 4 such doses were given over a period of 13 days. The

results are summarized in Figure 7. In no case did a recognizable cuticle appear until after the last dose was given. Apparently, the effects of the repeated doses are cumulative, and are independent of the dose given in a single exposure and of the time elapsed between the first and last exposure (Table I).

Discussion

Oberlander (1969a), working with wing discs of *Galleria*, reported that ecdysone remained active in cultures for as long as three days and found no evidence that the tissues metabolized appreciable quantities of the hormone. His findings and the results of the present experiments stand in sharp contrast to those of Shaaya (1969), Karlson and Bode (1969), and Ohtaki, Milkman, and Williams (1968), all of whom found that the half-life of ecdysone analogs in living insects was at best a few hours. The most obvious explanation for this difference between the *in vivo* and *in vitro* results is that the hormone is either rapidly inactivated or excreted by living insects; neither would occur to any great extent in an *in vitro* system. However, a recent study by Richman and Oberlander (1971) indicates that the situation may be more complex than this.

The fact that multiple doses of hormone will stimulate the production of multiple cuticles sheds some light on questions raised earlier by Marks and Leopold (1971) as to why the *in vitro* system produced cuticles containing only small amounts of procuticle. Since the cuticle produced by a second dose of hormone was essentially similar to the one laid down 12 to 15 days earlier, the mechanisms producing the cuticular materials must have retained the ability to operate *in vitro* over considerable periods. Moreover, since the explant was able to secrete as many as 5 similar cuticles, the incompleteness of the first one could not have occurred because of the inability of the explant to produce enough material. What occurred is apparently the normal response to ecdysterone stimulation, and the completion of cuticular deposition *in vivo* probably requires either an additional stimulus of a different type or another source of material. In any case, what occurred *in vitro* is only the first of a series of steps that leads to the formation of a complete cuticle, and this first step was repeated with each dose.

Oberlander (1969a, 1969b) found that a 2-hr exposure to ecdysone at a concentration of 3 μg/ml was sufficient to induce morphogenic changes in the wing discs of *Galleria*. In the present study, I found that the overall (7-day) threshold for cuticular deposition was 0.35 μgd, close to the 0.25 μgd required to induce morphogenesis in Oberlander's studies; with a 3-hr exposure, it was between 1.25 and 2.5 μgd. Thus, as the exposure decreased, the total dosage required to induce deposition increased, indicating that this system is somewhat less sensitive than Oberlander's. This lowering of the threshold with increasing exposures suggested that the accumulation of some substance or physiological event was taking place. Evidence that such a buildup did indeed occur was obtained from the experiments made with discontinuous doses. The subthreshold doses given over long periods eventually triggered a response; furthermore, when the total dose was the same, about the same response was obtained, whether the dosage was spread over 1, 5, or 13 days.

Since each chamber was washed three times and held for three days between exposures, it is difficult to believe that a quantitative buildup of the ecdysterone

occurred unless it was tightly bound by the tissue. Thus, the relationship between concentration and exposure and the quantitative accumulation of interrupted doses suggested that it was the "event" rather than a substance that accumulated. Like the "events" resulting from exposure to ionizing radiation, these are apparently discrete and of an all or none nature.

Ohtaki, Milkman, and Williams (1968), working with *Sarcophaga*, assayed the concentration of ecdysone present at various stages of development and found that it never reached the level required to induce pupation in an isolated abdomen, but it was never entirely absent. They concluded that the presence of a low titer of hormone over a period of time resulted in the accumulation of a series of covert events that accumulated until they finally produced an overt response. The minimum period for the process of accumulation, regardless of the concentration of hormone, was 8.5 hr, which is in the same range as the 3 to 12 hr reported by Oberlander (1969a, 1969b) and the 6 and 12 hr required for a 50% response in my experiments. These results give strong support to the hypothesis of Ohtaki, Milkman, and Williams (1968). Such an all or none event might be either the tenacious occupation by the ecdysterone molecule of a receptor site on or within the cell or the passage into the cell of a single molecule of the "macromolecular factor" proposed by Williams and Kambysellis (1969). In the latter case, the ecdysterone would act to mediate the passage of the molecule through the cell membrane.

Since the entire series of events leading to cuticle deposition can be induced in the same explant as many as 5 times, it is apparent that once deposition has been induced and the cells have discharged their cuticular materials, they also discharge their accumulated events and are ready to start a new cycle, again responding in the same way as untreated tissue.

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Summary

Quantitative studies were made of the stimulation of cuticular deposition in cockroach leg regenerates *in vitro* by the molting hormone ecdysterone. Preliminary experiments showed that the hormone remained active and that the tissues remained sensitive *in vitro* for as long as 30 days. Large doses given one week apart resulted in the production of multiple cuticles, but small doses given at shorter intervals gave a single, cumulative response. Time-dosage studies showed that the concentration of the hormone and the length of exposure make roughly equal contributions to the effect of the dose.

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